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Selective protection of renal tubular epithelial cells by heme oxygenase (HO)-1 during stress-induced injury

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Selective protection of renal tubular epithelial cells by heme oxygenase (HO)-1 during stress-induced injury.

Background. The renal pathology of human heme oxygenase (HO)-1 deficiency is characterized by advanced tubulointerstitial injury, whereas the glomerular structures are affected little. These facts suggest that the renal tubuli are dependent on intrinsic HO-1 production for their survival under oxidative stresses.

Methods. We compared the patterns of HO-1 expression by primary cultured human mesangial cells (HMCs) and renal proximal tubular epithelial cells (HRPTECs) in vitro. Furthermore, the cytoprotective roles of HO-1 induced in these cells were evaluated by stress-induced cytotoxicity assays. HO-1 expressions in HRPTECs and HMCs were evaluated by immunoblotting, and by reverse transcriptase (RT) and/or real time polymerase chain reaction (PCR).

Results. In HRPTECs, both HO-1 mRNA expression and protein production peaked at around 12 h and persisted until 24 h after hemin stimulation. In contrast, HO-1 mRNA expression and protein production by HMCs peaked at 4 h and 6 h respectively, and the levels declined rapidly, being undetectable at 24 h. The peak level of HO-1 expression was significantly higher in HRPTECs than in HMCs. Oxidative stress–induced cell injury in HRPTECs was significantly reduced when HO-1 production had been induced prior to the culture. In contrast, HO-1 induction had little cytoprotective effect on HMCs. Tin protoporphyrin (SnPP), an inhibitor of HO function, significantly reversed the cytoprotection by HO-1.

Conclusion. These data suggest that HRPTECs are more susceptible to oxidative stress and are significantly more dependent on HO-1 for protection against noxious stimuli than HMCs. Collectively, these results indicate that HO-1 is an important protective factor for kidney tissue, in particular, renal tubular epithelial cells.

Heme oxygenases (HOs) are rate-limiting enzymes that catalyze the conversion of heme into biliverdin, carbon monoxide, and iron [1–3]. Biliverdin is subsequently converted to bilirubin by biliverdin reductase and has potent antioxidant and anticomplement effects [4, 5]. Carbon monoxide acts as a potent vasodilator and inhibitor of platelet aggregation [6, 7]. Furthermore, degradation of heme by HOs leads to the induction of ferritin synthesis, which may then sequester free iron and prevent it from participating in subsequent oxidative injury [8–10]. Therefore, HOs have antioxidant capacity and act as potent anti-inflammatory proteins whenever oxidative injury takes place. They consist of three different isoforms. One is HO-1, which is an inducible form of the enzyme. The second is HO-2, which is a constitutive form of the enzyme expressed within the brain and testis. Recently, the third form, HO-3, has been reported as well [11].

We previously reported that the salient feature of the renal pathology of the first human case with heme oxygenase (HO)-1 deficiency was advanced tubulointerstitial injury [12, 13]. HO-1 staining in the human renal tissues was observed only within tubular epithelial cells and infiltrating macrophages in all renal diseases. In contrast, it was not detected within intrinsic glomerular cells, such as glomerular epithelial cells, glomerular endothelial cells, and mesangial cells. It is noteworthy that HO-1 staining tended to be more intense within distal than proximal tubuli. Furthermore, HO-1 staining was more intense with greater degrees of hematuria, presence of proteinuria, and tubulointerstitial damage within proximal tubuli [14]. In contrast, these differences were not evident within distal tubuli. These results suggest that HO-1 plays a pivotal role in the maintenance of renal functions by protecting the renal tubular epithelial cells under oxidative stress.

Contrary to these findings, in vitro studies have indicated that HO-1 is induced not only within tubular epithelial cells but also within mesangial cells, vascular endothelial cells, and various other cells. An important

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question is whether these differences reflect intrinsic differences in the HO-1–producing capacity of each cell type, or differences in the levels and characteristics of oxidative stresses which these cells encounter in vivo. To resolve this question, we compared human renal proximal tubular epithelial cells (HRPTECs) and human mesangial cells (HMCs) in vitro. Furthermore, stress-induced cytotoxicity assays were used to evaluate the cytoprotective roles of HO-1 induced in these cells.

METHODS

Cell culture

All reagents employed were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. The cultured HRPTECs (BioWhittaker, Inc., Walkersville, MD, USA) were maintained and subcultured in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM:F12) medium with 1 ng/mL hydrocortisone, 5 ng/mL insulin, 5 ng/mL transferrin, 5 pg/mL sodium selenite, 10 pg/mL epidermal growth factor, 6.6 ng/mL thyroxine, 50 ng/mL amphotericin-B, and 10% fetal calf serum (FCS). The cultured HMCs (BioWhittaker, Inc.) were maintained and subcultured in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY, USA) with 50 ng/mL amphotericin-B and 16% FCS. These cells were grown in a humidified incubator at 37°C and 5% CO_2 and studied as a confluent monolayer in all experiments. Concentrated stock of hemin was prepared freshly in 0.05 N sodium hydroxide and used to achieve the final concentration by appropriate dilution with the culture medium. Time course of hemin-induced HO-1 expression was examined as follows. Human renal proximal tubular epithelial cells and HMCs were precultured respectively in DMEM:F12 or RPMI-1640 medium containing 0.1% FCS (low-serum medium) for 24 hours before the induction of HO-1. The HO-1 production was induced by adding 20 μmol/L hemin to the culture medium and incubating at 37°C for 60 minutes. After removal of the hemin solution, these cells were further cultured in low-serum medium for various time periods. Preliminary experiments showed that little cell injury as detected by flow cytometry or lactate dehydrogenase (LDH) release was induced by 20 μmol/L hemin for up to 72 hours of culture (data not shown).

Immunoblotting

Samples for immunoblotting were prepared as described previously [12]. Briefly, equivalent numbers of cells were treated with 10% trichloroacetic acid (TCA) in phosphate-buffered saline (PBS) for 10 minutes on ice. The cells were spun down and suspended in 100 μL of lysis solution containing 9 mmol/L urea, 2% Triton X, and 10% 2-mercaptoethanol. The mixture was disrupted by sonication and neutralized with 1 mol/L Tris solution. The lysates were applied to precast sodium dodecyl sulfate (SDS) polyacrylamide gels, with 12 wells, 5 to 20% concentration, 10,000 to 200,000 molecular weight separation range (ATTO, Tokyo, Japan), and electrophoresis was carried out in SDS. The proteins were blotted onto nitrocellulose filters using a Horizblot apparatus (ATTO). Blots were blocked in 5% skim milk in PBS for 1 hour and reacted with rabbit anti-HO-1 and anti-HO-2 antiserum (StressGen, Victoria, British Columbia, Canada) or mouse anti-actin monoclonal antibody (Chemicon International, Temecula, CA, USA) diluted in PBS containing 0.1% Tween 20 (PBS-T) at optimal concentrations for 1 hour. The membranes were washed and further incubated with an appropriate dilution of peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG (Dako Co., Carpinteria, CA, USA) in PBS-T for 1 hour at room temperature. Immunoblots were developed by using enhanced chemiluminescence (ECL) signal generation system (Amersham Pharmacia Biotech, Inc., Buckinghamshire, UK) according to the manufacturer's instructions. A Lumi-Imager F1 (Roche Diagnostics Co., Ltd., Tokyo, Japan) was used to obtain the chemiluminescence image and analyses of the signals.

Reverse transcriptase PCR

Cells were detached by trypsinization after the culture, and total cellular RNA was isolated from 1×10^6 cells using TRIZOL Reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription was performed with random hexamer primer (Takara Shuzo Co., Tokyo, Japan) and RAV-2 reverse transcriptase (Takara Shuzo Co.). Amplification of cDNA by polymerase chain reaction (PCR) was performed with the following three separate sets of oligonucleotide primers, specific for human HO-1, HO-2, and β actin, respectively. HO-1: 5′-CGGCTTCAAGCTG GTGATG-3′ upstream, 5′-GGCTGGTGTGTAGGGG ATG-3′ downstream; HO-2: 5′-ACCTCAGAGGGGG TAGACG-3′ upstream, 5′-CCAGCTTAA ACAGCTC CTTC-3′ downstream; β actin: 5′-TGGACTTCGAGC AAGAGATG-3′ upstream, 5′-GATCTTCATTGTGCT GGGTG-3′ downstream. The PCR conditions were 94°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and finally additional extension at 72°C for 4 minutes using Gene Amp PCR system 9700 (PE Applied Biosystems, Japan Co., Tokyo, Japan).

Real-time PCR with a fluorogenic probe

The upstream and downstream sequences of PCR primers for the HO-1 real-time PCR are 5′-TGAGG AACTTTCAGAAGGGCC-3′ and 5′-TGTTGCGCTC AATCCCTCC-3′, respectively (Funakoshi Co., Ltd., Tokyo, Japan). A fluorogenic probe (5′-CGGCTT CAA GCTGGTGATGGCC-3′) with a sequence located be-

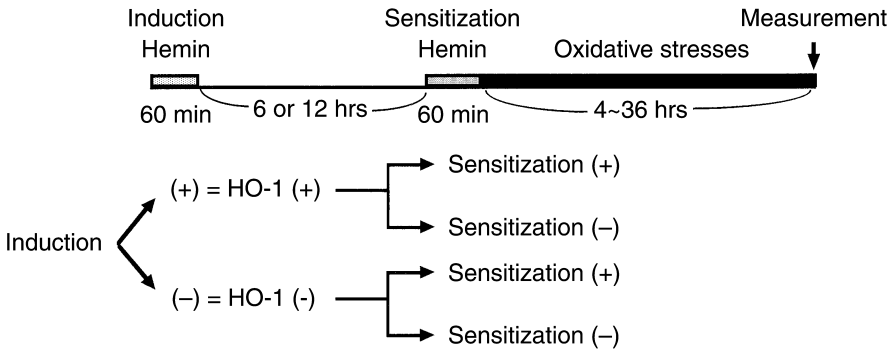

Fig. 1. Protocols of the cytotoxicity assay. The experimental protocols were subdivided into four different cultures. In half of the cultures, human renal proximal tubular epithelial cells (HRPTECs) and human mesangial cells (HMCs) were pulsed with 10 μmol/L of hemin prior to the cytotoxicity assays (induction culture). After removing the medium containing hemin, these cells were cultured for an additional 12 hours and 6 hours, respectively, to induce maximum heme oxygenase (HO)-1 production. After the induction cultures, one set each of these cells with or without induction cultures was exposed to 5 μmol/L of hemin for 1 hour to enhance oxidative stress-induced cytotoxicity (sensitization culture). Control cultures were performed without sensitization. Subsequently, all of these cells were incubated with different concentrations of various oxidative stresses.

tween the PCR primers was synthesized by PE Applied Biosystems. The PCR reaction was performed using the TaqMan PCR kit (PE Applied Biosystems Japan) according to the manufacturer's instructions. Briefly, 250 ng of DNA from each cell culture stimulated by hemin was added to a PCR mixture containing 10 mmol/L Tris (pH 8.3). KCl (50 mmol/L), 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 5.5 mmol/L $MgCl_2$, 100 μmol/L each of dATP, dCTP, dGTP, and dTTP, 0.6 μmol/L of each primer, 0.2 μmol/L fluorogenic probe, and 1.25 U of AmpliTaq Gold for 10 minutes at 95°C, 45 to 50 cycles of 15 seconds at 95°C, and 1 minute at 62°C were carried out by a model 7700 sequence detector (PE Applied Biosystems, Japan). Real-time fluorescence measurement was taken, and a threshold cycle (Ct) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit (10 times the standard deviation of baseline). For a positive control, a plasmid that contains HO-1 gene was constructed from pGEM-T vector (Promega, Madison, WI, USA). A standard graph of the Ct values obtained from serially diluted pGEM-HO-1 was constructed. The Ct values from samples were plotted on the standard curve, and the copy number was calculated automatically by Sequence Detector version 1.6 (PE Applied Biosystems, Japan), a software package for data analysis. Each sample was tested in duplicate, and the mean of the two values was shown as the copy number of the sample. Samples were defined as negative if the Ct values exceeded 50 cycles. GAPDH mRNA was quantitated simultaneously, and the HO-1/GAPDH ratio was calculated for each sample.

Cytotoxicity assays

The protocol of the cytotoxicity assay is shown in Figure 1. Four sets of cultures of HRPTECs and HMCs were prepared in 96-well plates at a density of 8×10^3 cells/100 μL per well and were cultured at 37°C for 36 hours. Two sets of the plates were pulsed with hemin by exchanging the culture medium with low-serum medium containing 10 μmol/L hemin and were cultured further for 1 hour (induction). The remaining plates were cultured without hemin stimulation. After the induction cultures, all of the plates were replaced with low-serum medium and incubated for an additional 12 hours or 6 hours in the case of HRPTECs or HMCs, respectively. These incubation times in the case of HRPTECs or HMCs are peak times of HO-1 expression. To accelerate the induction of cytotoxicity, one set each of the cultures with or without induction cultures were incubated with 5 μmol/L hemin for 1 hour before adding oxidative stresses (sensitization). Control cultures were continued without sensitization. Afterward, these cells were incubated with low-serum medium containing serial different concentration of H_2O_2 for 4 hours, cyclosporin A (CyA) (Novartis Pharma K.K., Tokyo, Japan) for 4 hours, gentamicin (GM) (Schering-Plough Co., Osaka, Japan) for 4 hours, cisplatin (Bristol Myers Squibb K.K., Tokyo, Japan) for 12 hours, cadmium chloride (Cd) (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) for 24 hours, or $NaAsO_2$ (As) (Wako Pure Chemical Industries, Ltd.) for 36 hours. Kinetics of cytotoxicity was evaluated by preliminary experiments, and the optimal culture period for each reagent was determined. Viabilities of these cells were determined by Cell Counting kit 8 (Wako Pure Chemical Industries, Ltd.) after the cultures, as described previously [15]. Briefly, the plates were washed with PBS, fresh culture medium was added to each well, and 10 μL of the reaction mixture containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was added to each well. After 2 hours' incubation under 5% CO_2 at 37°C, the solubilized formazan product was quantified spectrophotometrically using a microplate reader. To determine if the cytoprotective effects of induction cultures were mediated by hemin-induced HO-1, HPRTECs were

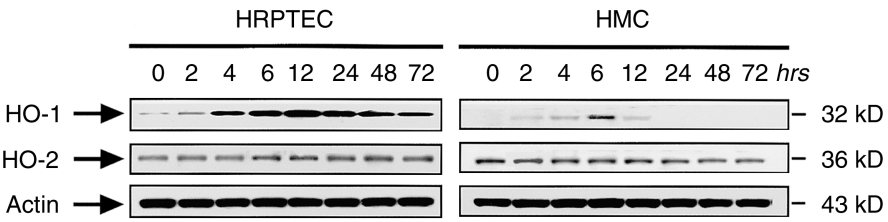

Fig. 2. Immunoblotting of heme oxygenase (HO)-1 and HO-2 induced in human renal proximal tubular epithelial cells (HRPTECs) and human mesangial cells (HMCs). HO-1 production was induced by hemin stimulation in HRPTECs (left) and in HMCs (right). The expression of HO-2 within HRPTECs and HMCs was constitutive. Lanes 1 through 8 indicate samples at 0, 2, 4, 6, 8, 12, 24, and 72 hours after hemin stimulation. Actin served as control protein. Results shown are representative of five separate experiments.

exposed to 75 μmol/L tin protoporphyrin (SnPP) (Affiniti Research Products, Ltd., Exeter, UK), a potential inhibitor of heme oxygenase, before the cytotoxicity assay.

Statistical analysis

The data were shown as mean ± SEM for each group. Statistical analysis was performed by one-way analysis of variance (ANOVA) or by unpaired *t* test. All results were considered statistically significant at $P < 0.05$.

RESULTS

HO-1 expression in cultured renal cells

HO-2 protein was constitutively expressed in both HRPTECs and HMCs without hemin stimulation, and the levels did not change significantly for 72 hours after hemin stimulation. In contrast, HO-1 was detectable only at very low level in HRPTECs before the culture. The level increased progressively after 2 hours, peaked at 12 hours, and remained high even after 72 hours (Fig. 2, left panel). In HMCs, HO-1 was not detectable before the culture. It was delectable at 4 hours, peaked at 6 hours, and the level decreased rapidly thereafter (Fig. 2, right panel). HO-1 mRNA levels were compared to see if the differences in HO-1 production by these two cell lines reflect differences in de novo HO-1 gene transcription. HO-1 mRNA was detectable in both cell lines shortly after stimulation. In HRPTECs, HO-1 mRNA levels continued to increase and peaked at 12 hours. Surprisingly, a significant level of HO-1 mRNA remained detectable even at 24 hours. In marked contrast, HO-1 mRNA levels increased rapidly, peaked at 4 hours, and decreased sharply in HMCs. It was not detectable at 24 hours (Fig. 3). HO-2 and β-actin mRNAs were detectable before stimulation and remained constant throughout the cultures in both cell lines.

Cytotoxicity assays of HRPTECs and HMCs

The viability of HRPTECs was significantly higher after hemin-pulsed induction of HO-1 than control HRPTECs at H_2O_2 concentration, 250 and 500 μmol/L (94.8 ± 4.55% vs. 76.3 ± 5.36% and 56.9 ± 6.73% vs.

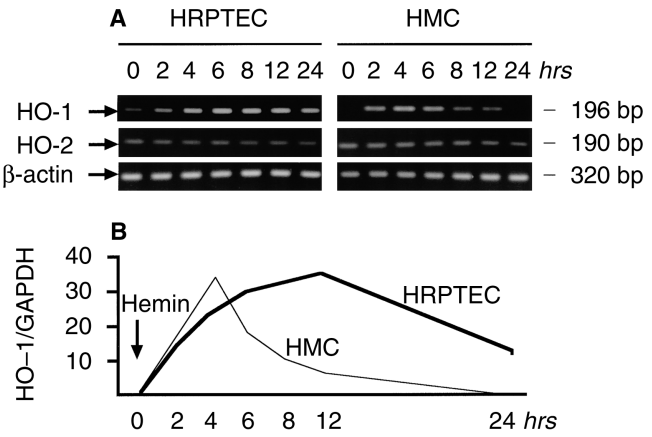

Fig. 3. Kinetics of heme oxygenase (HO)-1 and HO-2 mRNA expressions. (*A*) Sequential changes in the expression of HO-1 and HO-2 mRNAs in human renal proximal tubular epithelial cells (HRPTECs) (left) and human mesangial cells (HMCs) (right) were compared by reverse transcriptase polymerase chain reaction (PCR). The levels of β-actin served as control. (*B*) Furthermore, the levels of HO-1 mRNA were quantified by real time PCR. Degrees of HO-1 mRNA expression by HRPTECs (bold line) and HMCs (thin line) are expressed as HO-1/GAPDH ratio. Horizontal axis indicates the time after hemin treatment.

19.8 ± 2.92%, respectively) (Fig. 4A). In contrast, the induction culture with hemin had little effect on the survival of HMCs (Fig. 4C). The differences in the effects of the hemin-pulsed induction were further enhanced by sensitization of these cell lines with a low dose of hemin prior to the addition of H_2O_2 (Fig. 4 B and D). Cell viability decreased dramatically when HRPTECs were sensitized before the addition of H_2O_2. Cell injury was observed with a much lower dose of H_2O_2 than the culture without sensitization. It is intriguing that the cell injury was inhibited significantly by the hemin-pulsed induction culture (Fig. 4B), whereas little significant changes in cell viability were observed in HMC cultures (Fig. 4D). Although HRPTECs were less sensitive to H_2O_2 without sensitization, they became significantly more sensitive to H_2O_2 than HMCs after the sensitization culture.

Next, we examined whether hemin-pulsed induction has any cytoprotective effect on HRPTEC cultures regardless of the nature of the oxidative stresses imposed.

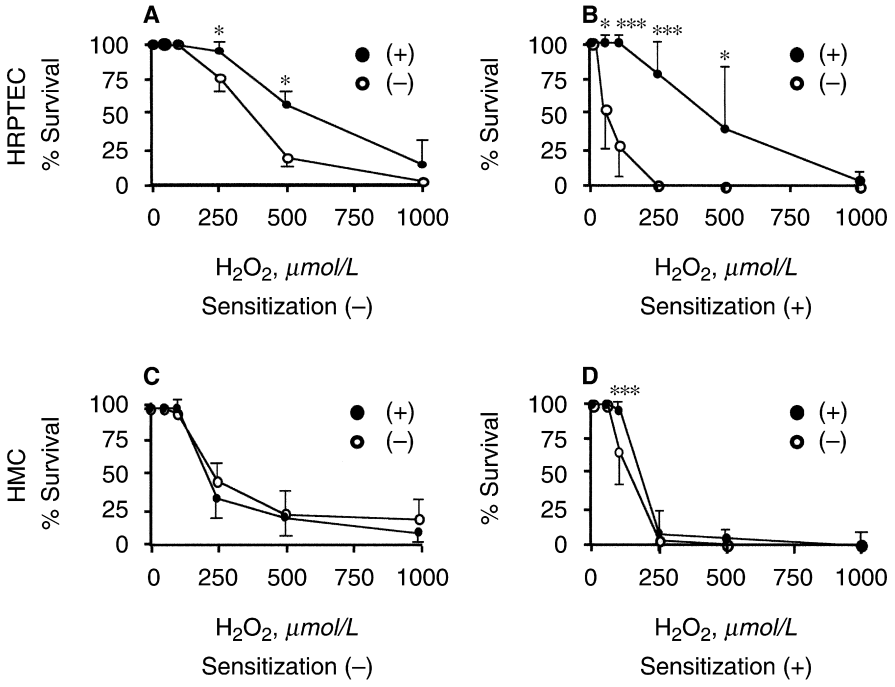

Fig. 4. Effects of induction and sensitization cultures on cytotoxicity assays. Viability of human renal proximal tubular epithelial cells (HRPTECs) and human mesangial cells (HMCs) was evaluated after the cultures with different concentrations of H_2O_2. Cells were cultured (●) with or (○) without prior hemin-pulsed cultures. (*A*) HRPTECs without sensitization; (*B*) HRPTECs with sensitization; (*C*) HMCs without sensitization; and (*D*) HMCs with sensitization. Results are expressed as mean ± SEM ($N = 5$). Statistical significance was evaluated between cultures with or without prior hemin-pulsed cultures. *$P < 0.05$; ***$P < 0.001$.

All the assays were performed with prior sensitizations. Cell injury of HRPTECs induced by 250 μmol/L H_2O_2 was significantly reversed by prior hemin-pulsed culture, whereas the same treatment had no effect on HMC survival (Fig. 5A). Cyclosporin (CyA)-induced cell injury of HRPTECs was similarly inhibited by hemin-pulsed culture while having no effect on HMCs (Fig. 5B). Gentamicin (GM) and cisplatin (Cis) induced less significant levels of cytotoxicity in HRPTECs and little cell injury in HMCs. Nevertheless, the effect of the hemin-pulsed induction culture was similarly observed in HRPTECs (Fig. 5 C and D). Less significant, but identical patterns of cytoprotection by the hemin-pulsed culture were observed when HRPTECs were cultured with cadmium chloride (Cd) and $NaAsO_2$ (As) (Fig. 5 E and F).

We next tried to exclude the possibility that these differences reflect differences in the donors of HRPTECs and HMCs rather than intrinsic differences of the cell origins. For this purpose, we repeated the experiments using different HRPTECs and HMCs from three unrelated donors. As shown in Figure 6, HRPTECs and HMCs from different donors showed essentially identical results.

Effect of HO inhibition on hemin-induced cytoprotection

To confirm the cytoprotective role of HO-1 in oxidative stress–induced cell injury, we employed a specific competitive inhibitor tin protoporphyrin (SnPP). In control cultures, survival of HRPTECs was significantly increased when the cells had been pulsed with hemin (4.9 ± 1.5% vs. 89.1 ± 5.1%, $P < 0.001$). However, the effect of hemin-pulsed induction was almost completely abolished when SnPP was added to the cytotoxicity assay (45.5 ± 5.4% vs. 50.6 ± 5.9%) (Fig. 7). SnPP alone increased survival of HRPTECs to a certain degree. Addition of SnPP had little effect on the survival of HMCs (data not shown).

DISCUSSION

Tubulointerstitial injury can impair renal function through multiple pathways. It is a pathologic process consistently present in various kidney diseases. Tubulointerstitial injury is characterized by tubular atrophy, enhanced interstitial fibrosis, and deposition of extracellular matrix. It has been reported that renal functional impairment correlated more closely with tubulointerstitial changes than with glomerular injuries in all renal diseases [16]. We previously reported that HO-1 was detected within tubular epithelial cells, but not within intrinsic glomerular cells in various renal diseases [14]. In the first reported human HO-1 deficiency case, tubulointerstitial damage progressed, resulting in tubular obstruction and secondary enlargement of Bowman's capsules [13]. We speculated that HO-1 is a critical factor that protects renal tubular epithelial cells from oxidative injury and prevents progression to renal failure. However, we do not know at present the functional significance of the particular anatomic distribution of HO-1 within the kidney. We need to elucidate if the unique pattern of HO-1 expression reflects intrinsic differences in the capacity of distinct cell types to produce HO-1 in response to oxidative stress, or if they are the result of different levels

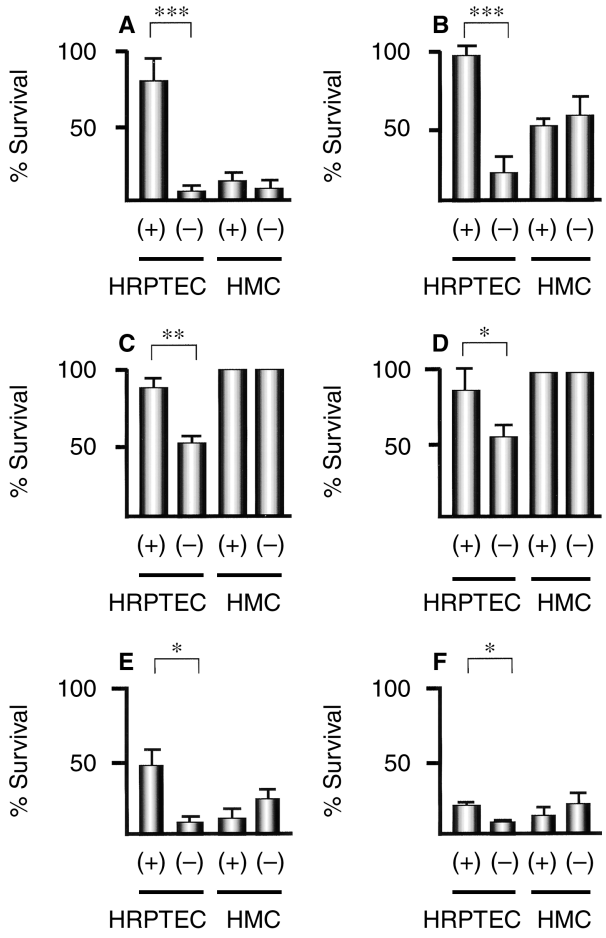

Fig. 5. Effect of hemin-pulsed cultures on viability of human renal proximal tubular epithelial cells (HRPTECs) and human mesangial cells (HMCs) after stimulation with various oxidative stresses. Cells with (+) or without (−) prior hemin-pulsed cultures were exposed to 5 μmol/L hemin (sensitization) for 1 hour and stimulated with (*A*) 250 μmol/L H_2O_2, (*B*) 2.5 μg/mL cyclosporine (CyA), (*C*) 40 μg/mL gentamicin (GM), (*D*) 25 μg/mL cisplatin (Cis), (*E*) 30 μmol/L cadmium chloride (Cd), or (*F*) 30 μmol/L sodium arsenite (As). Results are expressed as mean ± SEM ($N = 5$). Statistical significances were evaluated between cultures with or without prior hemin-pulsed cultures. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

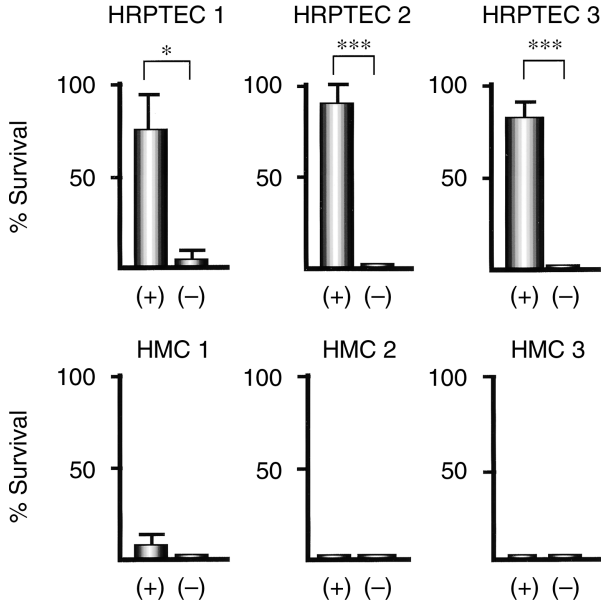

Fig. 6. Protective effects of hemin-pulsed cultures on H_2O_2-induced cytotoxicity of human renal proximal tubular epithelial cells (HRPTECs) and human mesangial cells (HMCs) derived from different individuals. H_2O_2-induced cytotoxicity assays were repeated (+) with or (−) without prior hemin-pulsed cultures using primary culture cells from three distinct individuals. Results are expressed as mean ± SEM ($N = 5$). Statistical significance was evaluated between cultures with or without prior hemin-pulsed cultures. $^{***}P < 0.001$.

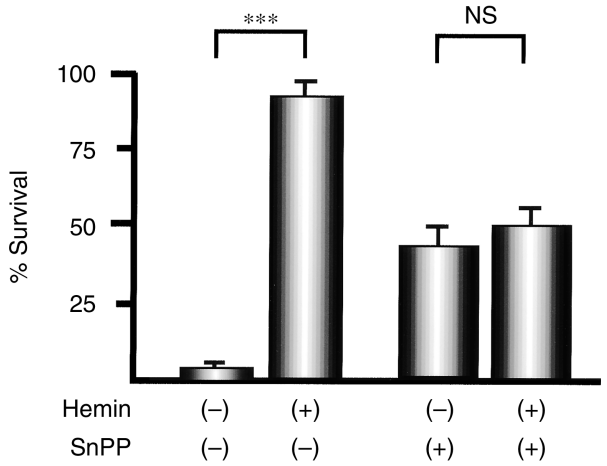

Fig. 7. Abrogation of cytoprotective effects of heme oxygenase (HO)-1 by tin protoporphyrin (SnPP). SnPP was added to human renal proximal tubular epithelial cell (HRPTEC) cultures (+) with or (−) without prior hemin-pulsed cultures, and the cells were exposed to 100 μmol/L H_2O_2. Results are expressed as mean ± SEM ($N = 5$). Statistical significance was evaluated between cultures with or without prior hemin-pulsed cultures. $^{***}P < 0.001$.

of oxidative stress encountered in vivo by these cells. To provide answers to these questions, we utilized primary cultures of HMCs and HRPTECs and examined the patterns of expression and the functional significance of HO-1 in the two kinds of cultured human renal cells.

The immunoblotting data showed that HRPTECs produce significantly larger amounts of HO-1 than HMCs (Fig. 2) in response to hemin stimulation. Not only was the peak expression of HO-1 by HRPTECs higher than that by HMCs, but the duration of HO-1 production in HRPTEC cultures was significantly longer than that in HMC cultures as well, indicating that HRPTECs produce substantially greater levels of HO-1 than HMCs under oxidative stress. These differences in HO-1 production are attributable to differences in de novo HO-1 gene transcriptions, as indicated by the quantitative HO-1 mRNA measurements shown in Figure 3. These data support the notion that tubular epithelial cells have an intrinsic capacity to produce HO-1 upon stimulation, much more efficiently than other components of the kid-

ney, including the mesangial cells. The data also explain the unique distribution of HO-1 producing cells within the kidney in vivo. Preferential HO-1 expression by the renal tubules is at least partly explained by the inherent capacity of these cells to produce HO-1. We next addressed the issue of whether the characteristic pattern of HO-1 production has any functional significance under oxidative stress.

Accumulating evidence has demonstrated that the reactive oxygen metabolites play a role in the pathogenesis of various renal diseases. Moreover, the levels of tissue injury and organ dysfunction are regulated by the balance between the degree of reactive oxygen metabolites and the amount of scavengers of the oxidative stresses [17–20]. H_2O_2 is produced as a result of a very active oxidative metabolism. In this particular study, we examined the effect of prior HO-1 induction on H_2O_2–mediated cell injury of HRPTECs and HMCs. The results demonstrated that prior HO-1 induction by hemin-pulse resulted in a marked increase in cell viability in HRPTEC cultures. In contrast, hemin-pulsed induction had little significant effect on the viability of HMCs, largely due to the fact that HMCs produced only a limited amount of HO-1 for a short duration upon stimulation.

In the cytotoxicity assays, cells were exposed to hemin for a short period of time before the cultures. It was observed that hemin-pulsed induction cultures were cytoprotective, whereas short-term exposure to hemin augmented the cell injury, indicating the presence of time-dependent dichotomous effects of hemin exposure on target cells. Active heme metabolism may increase apparent oxidant sensitivity in the short-term culture by creating a transient flux in intracellular chelatable iron, whereas it may produce a marked net antioxidative effect by coupling with the iron sequestration pathway [8, 9, 21, 22].

Because we used the primary cultures of HRPTECs and HMCs for these experiments, it was necessary to confirm that the distinct patterns of HO-1 production by these two types of primary cultured renal cells reflect the intrinsic differences of the two lineages rather than differences attributable to the cell donors. The results shown in Figure 6 clearly indicate that HRPTECs are dependent on induced HO-1 for cell survival, regardless of the cell source.

The effect of hemin-pulse was due to the induction of HO-1, as the coculture with specific competitive inhibitor of HO-1, SnPP cancelled the cytoprotective effect of hemin-pulsed cultures. Moreover, SnPP alone enhanced the survival of sensitized HRPTECs. The results correlate with the finding of transiently increased ferritin concentration by HO inhibitor [10], and the suggestion that the increased ferritin may sequester a transient flux-free iron derived from sensitized HRPTECs to decrease the oxidative cell injury. It is known that renal proximal tubular epithelial cells play a major role in reabsorptive function and display a markedly high rate of oxygen consumption. Therefore, it is the site of the most intensive oxidation processes. H_2O_2 and other reactive oxygen metabolites may contribute to the injury of renal proximal tubular epithelial cells and to renal fibrosis, unless effective cytoprotective mechanisms play roles. We speculate that HO-1 is one such agent.

To further confirm our hypothesis, we employed other nephrotoxic agents that are known to induce proximal tubular necrosis, including gentamicin, cisplatin, cyclosporin, and heavy metals. Regardless of the type of oxidative stress, the viability of HRPTECs improved significantly when HO-1 was induced by hemin stimulation. In contrast, HO-1 expression had no significant effect on the viability of HMCs. There has been accumulating evidence of a role for reactive oxygen metabolites in various renal diseases including toxic acute renal damage caused by gentamicin, cyclosporin, and cisplatin. Iron and H_2O_2 induced tissue injury by catalyzing free radical formation. Scavengers of reactive oxygen metabolites such as hydroxyl radical scavengers and iron chelators provide protection in nephrotoxicity [17, 23–28]. Nephrotoxicity of heavy metals such as cadmium, arsenite, mercury, and lead might be mediated via tissue damage elicited by the oxidative stresses. To avoid rapid cell injury induced by high dose of heavy metals, we used long exposure times with low doses of As or Cd. Therefore, it is possible that the low-dose heavy metals themselves induced high levels of HO-1 during the long incubation period, sufficiently enough to protect HRPTECs. This may explain why hemin-pulsed cultures showed little cytoprotective effect on heavy metal-induced cytotoxicity of HRPTECs. Whatever the major source of protective HO-1, our experimental results may sufficiently support the view that localized production of HO-1 protects renal tubular epithelial cells from the oxidative stresses induced by the heavy metals [29–32]. As stated above, protecting renal tubular cells against oxidant injury is critical to prevent renal progressive damage.

A series of studies also showed that prior induction of HO-1 drastically prevented progression of renal failure [27, 28, 31, 33, 34]. Agarwal et al [35] reported that SnPP prevented the induction of heme oxygenase and consequently led to higher serum creatinine values and exacerbation of renal injury in a rat cisplatin-nephropathy model. Our data also indicate that the cytoprotective effect of hemin-stimulation is through HO-1 induction in the target cells. Collectively, these data show the importance of HO-1 induction both in vivo and in vitro in the protection of renal tubular cells and preservation of the renal functions in the event of overwhelming oxidative stresses.

CONCLUSION

The present study indicated that tubular epithelial cells are capable of producing HO-1 much more efficiently than mesangial cells, with the cytoprotective effect of HO-1 thereby playing a significant role in vivo. These studies offer hope that modulation of HO-1 gene expression may provide a new therapeutic modality to prevent or limit progressive renal damage in human.

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